Appl Microbiol Biotechnol (1998) 50: 323–330

ORIGINAL PAPER

Y. Nagasaka · K. Kurosawa · A. Yokota · F. Tomita

Purification and properties of the raw-starch-digesting glucoamylases from *Corticium rolfsii*

Received: 16 December 1997 / Received last revision: 6 May 1998 / Accepted: 1 June 1998

Abstract *Corticium rolfsii* AHU 9627, isolated from a tomato stem, is one of the strongest producers of a raw-starch-digesting amylase. The amylase system secreted by *C. rolfsii* AHU 9627 consisted of five forms of glucoamylase (G1–G5) and a small amount of α-amylase. Among these amylases, G1, G2 and G3 were able to hydrolyze raw starch. Five forms of glucoamylase were separated from each other and purified to an electrophoretically homogeneous state. The molecular masses were: G1 78 kDa, G2 78 kDa, G3 79 kDa, G4 70 kDa, and G5 69 kDa. The isoelectric points were: G1 3.85, G2 3.90, G3 3.85, G4 4.0, and G5 4.1. These glucoamylases showed nearly identical characteristics except that G4 and G5 were unable to hydrolyze raw starch.

Introduction

From the point of view of energy saving, the enzymatic saccharification of raw starch in a non-cooking system is desirable. In the previous papers, we reported the discovery of a raw-starch-digesting enzyme from *Corticium rolfsii* AHU 9627 (Sasaki et al. 1986), the optimization of the culture conditions for the enzyme production (Takao et al. 1986) and some applications of the crude enzyme (Kurosawa et al. 1989; Hariantono et al. 1991). In the preceding paper (Nagasaka et al. 1995), cDNA cloning of a raw-starch-digesting glucoamylase G2, one of the components of the amylase system of *C. rolfsii* AHU 9627, was reported, and the primary structure of this enzyme was compared with that of the raw-starch-digesting glucoamylases from other microorganisms, e.g., *Aspergillus niger* (Boel et al. 1984), *A. oryzae* (Hata et al. 1991), *Hormoconis resinae* (Vainio et al. 1993), *Humicola grisea* var. *thermoidea* (unpublished results), *Neurospora crassa* (Stone et al. 1993), *Rhizopus oryzae* (Ashikari et al. 1986). This paper reports on the constitution of the amylase system of *C. rolfsii* AHU 9627 and some properties of the glucoamylases purified (G1–G5). Kaji et al. (1976) described an acid-stable glucoamylase from *C. rolfsii* IFO 4878. However, the enzyme was not sufficiently characterized and they did not refer to the action on raw starch.

Y. Nagasaka · K. Kurosawa[1] · A. Yokota · F. Tomita (✉)
Laboratory of Applied Microbiology,
Department of Bioscience and Chemistry,
Faculty of Agriculture, Hokkaido University,
Sapporo 060, Japan
Fax: +81 11 706 4961

Present address:
[1]Research Institute of Life Science,
Snow Brand Milk Products Co. Ltd.,
Tochigi 329-05, Japan

Materials and methods

Preparation of a crude enzyme

C. rolfsii AHU 9627 was cultivated in a basal medium at 27 °C for 10 days as described in a previous paper (Takao et al. 1986). To the culture supernatant was added ammonium sulfate to 80% saturation. After the culture had been allowed to stand for 24 h, the precipitate was collected by vacuum filtration through a thick bed of Celite 545 (Kanto chemical Co. Inc., Tokyo, Japan) over a glass-fibre filter-paper (GD 120, Toyo roshi kaisha Ltd., Tokyo, Japan), and dissolved in a small amount of deionized water. Unless otherwise specified, the following purification procedures were carried out at 4 °C. The solution was applied on a Bio-gel P-6 (Bio-Rad Laboratories, Richmond, Calif., USA) column (2.4 × 40 cm), equilibrated with 10 mM sodium acetate buffer (pH 5.0). The protein fraction recovered was used as a crude enzyme.

Partial purification of amylase components

The crude enzyme solution was applied on a column of DEAE-Toyopearl 650 M (Tosoh Corporation, Tokyo, Japan, 2.0 × 18 cm) equilibrated with 10 mM sodium acetate buffer (pH 5.0). The protein was eluted with a linear gradient of 0–0.25 M NaCl in the same buffer.

Corticium protease preparation

After elution of the amylase components, as described in the preceding section, the DEAE-Toyopearl 650 M column was eluted with 1.0 M NaCl solution. The eluate was used as a *Corticium* protease preparation.

Purification of glucoamylases

In the following purification steps, a Bio-gel P-6 column (2.4 × 40 cm) was used for desalting and changing the buffer of the enzyme solution.

The G1 fraction, obtained by a partial purification, was desalted and the buffer was changed to 10 mM sodium acetate buffer pH 4.3. The resulting G1 fraction was applied on a DEAE-Sepharose CL-6B (Tosoh Corporation) column (2.0 × 18 cm) equilibrated with the above buffer, and eluted by a linear gradient of 0–0.1 M NaCl in the same buffer. The eluted enzyme fraction was desalted and the buffer was changed to 10 mM sodium acetate buffer pH 4.0. The resulting enzyme solution was rechromatographed on a DEAE-Toyopearl 650 M column (2.0 × 18 cm) using the same gradient system.

The G2 fraction, separated by the partial purification, was purified by the same method as used for G1.

The G3 fraction obtained by the partial purification was desalted and the buffer was changed to 10 mM sodium acetate buffer pH 4.3. The resulting G3 fraction was applied on a DEAE-Toyopearl 650 M column (2.0 × 18 cm) equilibrated with the above buffer, and eluted by a linear gradient of 0–0.1 M NaCl in the same buffer. The eluted G3 fraction was desalted and the buffer was changed to modified McIlvaine buffer pH 3.0 (Mixture of 10 mM citric acid solution and 20 mM disodiumhydrogenphosphate solution). The resulting enzyme solution was chromatographed on a SP-Toyopearl 650 M (Tosoh Corporation) column (2.0 × 18 cm) using the same gradient system. Finally, the eluted enzyme fraction was desalted and the buffer was changed to 10 mM sodium acetate buffer pH 4.3. The resulting enzyme solution was chromatographed on a DEAE-Sepharose CL-6B column (2.0 × 18 cm) using the same gradient system.

The glucoamylase fraction containing no activity on raw starch, obtained by partial purification, was desalted and the buffer was changed to modified McIlvaine buffer pH 3.0. The resulting enzyme fraction was applied on a SP-Toyopearl 650 M column (2.0 × 18 cm), equilibrated with the above buffer and eluted by a linear gradient of 0–0.1 M NaCl in the same buffer. The eluted enzyme fraction was desalted and buffer was changed to 25 mM piperazine/HCl buffer (pH 5.5). The resulting fraction was applied on a Chromatofocusing column (PBE 94, Pharmacia, Uppsala, Sweden, 1.3 × 16 cm) equilibrated with the above buffer and eluted by a gradient of pH 5.0–4.0 in Polybuffer/HCl (pH 4.0). G4 and G5 were eluted at pH 4.5 and pH 4.2 respectively. To remove carrier ampholyte, the G4 and G5 fractions were precipitated by ammonium sulfate and gel-filtered in 10 mM sodium acetate buffer pH 4.0.

Enzyme activity

Glucoamylase activity

The reaction mixture, containing 0.25 ml 1% (w/v) cooked soluble starch, 0.2 ml 0.1 M sodium acetate buffer (pH 4.0) and 0.05 ml enzyme solution, was incubated at 30 °C for 30 min. The glucose liberated into the supernatant was measured by the glucose-oxidase method (Glucose-ARII, Wako Pure Chemical Industries Ltd., Osaka, Japan). One unit (U) of glucoamylase activity was defined as the amount of the enzyme that liberated one micromole of glucose per minute.

Maltase activity

Maltase activity was assayed by the same method as glucoamylase activity but the substrate was replaced by maltose.

Raw- and gelatinized-starch-saccharifying activities

The methods for assaying raw- and gelatinized-starch-saccharifying activities were described previously (Nagasaka et al. 1995).

Protease activity

Protease activity was determined by incubating 0.2 ml enzyme solution with 1 ml 0.6% Hammarsten's casein (buffered at pH 2.5) for 30 min. One unit (U) of protease activity was defined as that which converted the substrate to one microgram of tyrosine in one minute at 30 °C, measured by Folin's procedure.

Determination of protein

The protein concentration was measured by UV adsorption at 280 nm.

Electrophoresis

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 5% gel by the method of Weber and Osborn (1969), and on an 8% gel by the method of Laemmli (1970). The gels were stained with Coomassie brilliant blue R-250.

The isoelectric point (pI) was determined using Pharmacia-LKB ampholytes as described by Vesterberg (1971).

Carbohydrate content

The carbohydrate contents were estimated by the phenol sulfate method with maltose as standard.

Determination of anomeric configuration

Anomeric configuration was determined using gas chromatography by the method of Chiba et al. (1983).

Raw-starch-digesting ability

The ratio of raw-starch-saccharifying activity to gelatinized-starch-saccharifying activity, was estimated as described in a previous paper (Nagasaka et al. 1995) but the substrate for the raw-starch-saccharifying activity was replaced by various kinds of raw starch.

Raw starch adsorption

Enzyme solution was incubated with each of the buffers containing 1% (w/v) raw corn starch at 4 °C for 10 min. The glucoamylase activity of the supernatant was then measured.

Kinetics

The reaction mixture, containing 1.0 ml substrate solution, 0.8 ml 0.1 M sodium acetate buffer (pH 4.5) and 0.2 ml enzyme solution, was incubated at 30 °C. The glucose liberated from each substrate was measured by Glucose-ARII and the K_m and V values were estimated from Lineweaver-Burk plots.

Amino acid sequence analysis

The N-terminal amino acid sequence of the protein was analyzed with a gas-phase sequencer (model 477A-120A, Perkin-Elmer Applied Biosystems Division, Foster City, Calif., USA). The C-terminal amino acid sequence was determined by analysis of the amino acids liberated with carboxypeptidase Y.

Amino acid composition

The amino acid compositions were determined with a derivatizer (model 420A-130A, Perkin-Elmer Applied Biosystems Division, Foster City, Calif., USA) after hydrolysis by 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole at 110 °C for 24 h.

Results

Partial purification of amylase components

According to the procedures described in Materials and methods, the crude enzyme solution was applied on a column of DEAE-Toyopearl 650 M. The elution patterns of the enzyme activities and the absorbance at 280 nm are shown in Fig. 1. The amylase system secreted by *C. rolfsii* AHU 9627 consisted of five forms of glucoamylase (G1–G5) and a small amount of α-amylase (fraction 63). Among these enzymes, G1, G2 and G3 were able to hydrolyze raw starch.

Purification of glucoamylases

According to the procedures described in Materials and methods, glucoamylases G1–G5 were purified to an electrophoretically homogeneous state (Fig. 2). A summary of the purification procedure is given in Table 1.

pH optimum and pH stability

The optimum reaction pH was investigated by measuring enzyme activity in various pH conditions at 30 °C. pH stability was investigated by measuring the remaining activity of the enzyme after it had been kept for 24 h in various pH conditions at 30 °C. Figure 3 shows the pH optimum and pH stability of glucoamylase G1. The five glucoamylases showed the same results for pH optimum and pH stability for activity on gelatinized starch. G1, G2 and G3 had the same pH optimum and stability for activity on raw starch. The pH optimum for raw starch was 4.0, whereas that for gelatinized starch was more alkaline, from around 4.5 to 5.5. Such a shift in pH optimum was also seen in the glucoamylases from black *Aspergillus* (Medda et al. 1982), *Endomycopsis fibuligera* (Ueda and Saha 1983) and *Rhizopus* sp. (Takahashi et al. 1985). Five glucoamylases were stable over a pH range of 3.0–8.0.

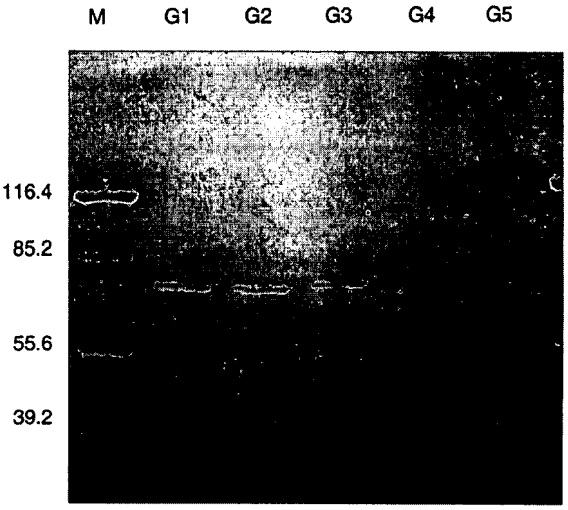

Fig. 2 Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of the purified glucoamylases (G1, G2, G3, G4 and G5)

Optimum temperature and thermostability

The optimum temperature was measured in 0.1 M sodium acetate buffer (pH 4.0) at various temperatures,

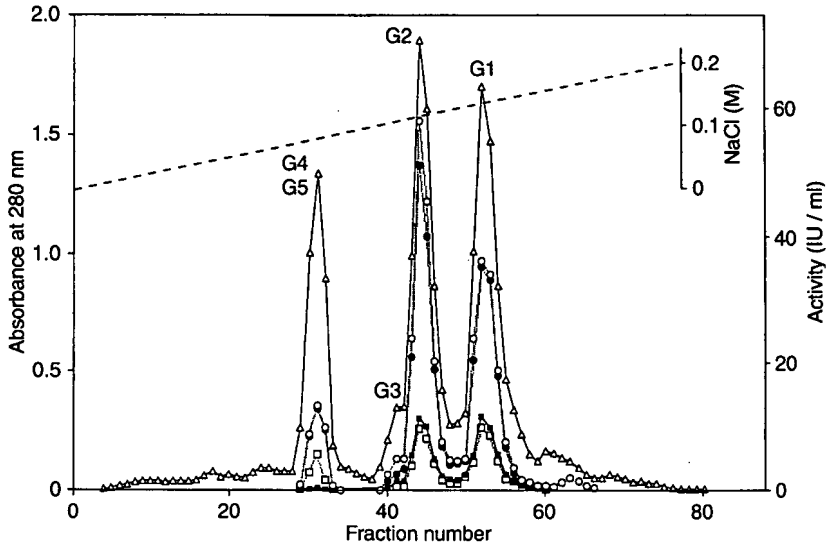

Fig. 1 DEAE-Toyopearl 650 M column chromatography of a crude enzyme. Column size: 2.1 × 13 cm. Fraction volume: 8 ml. Equilibration: 10 mM acetate buffer (pH 5.0). Elution: linear gradient of NaCl (0–0.25 M). Flow rate: 30 ml/h. △ Absorbance at 280 nm, ■ raw-starch-hydrolyzing activity, ○ gelatinized-starch-hydrolyzing activity, ● glucoamylase activity, □ maltase activity

Table 1 Summary of the purification of glucoamylases G1–G5

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Culture filtrate (300 ml)	4170[a]	5190	1.2	100
$(NH_4)_2SO_4$ precipitate	596[a]	3556	6.0	69
DEAE-Toyopearl 650 M (1st)				
G1	28	988	35	19
G2	26	835	32	16
G3	7.7	283	37	5.5
G4, G5	26[a]	264	10	5.1
G1				
DEAE-Sepharose CL-6B	24	920	38	18
DEAE-Toyopearl 650 M (2nd)	21	854	41	16
G2				
DEAE-Sepharose CL-6B	21	724	35	14
DEAE-Toyopearl 650 M (2nd)	17	660	39	13
G3				
DEAE-Toyopearl 650 M (2nd)	2.8	118	42	2.3
SP-Toyopearl 650 M	2.0	82	41	1.6
DEAE-Sepharose CL-6B	1.7	64	38	1.2
G4 and G5				
SP-Toyopearl 650 M	6.2[a]	185	29	3.6
Chromatofocusing				
G4	0.7	31	44	0.6
G5	2.0	94	47	1.8

[a] These values were calculated on the assumption that $A_{1\,cm}^{1\%}$ at 280 nm was 10, and other values on the basis that $A_{1\,cm}^{1\%}$ at 280 nm was 17.8 (G1), 18.0 (G2), 18.1 (G3), 17.7 (G4) and 18.0 (G5)

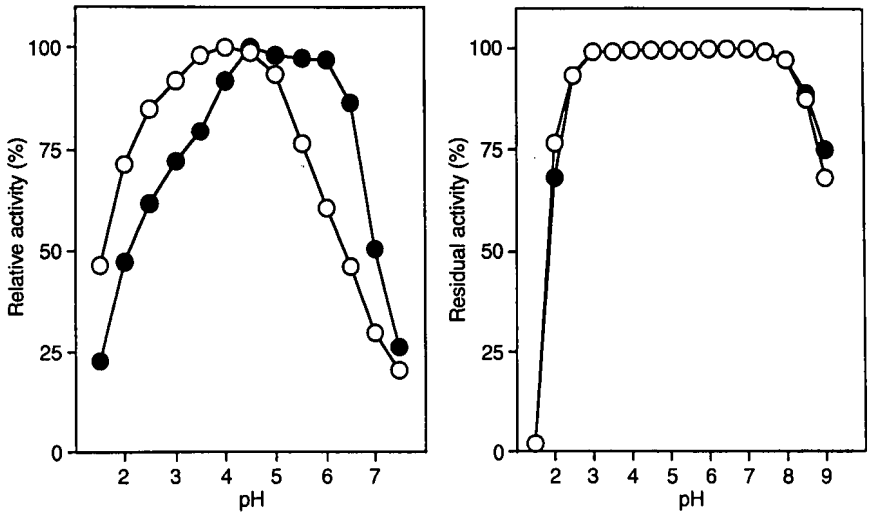

Fig. 3 Effect of pH on the activity (*left*) and the stability (*right*) of glucoamylase G1. Buffers used were 0.1 M HCl/KCl (pH 1.5–2.0), 0.1 M McIlvaine (pH 2.5–7.0) and TRIS/HCL (pH 7.5–9.0). ○ Raw-starch-hydrolyzing activity, ● gelatinized-starch-hydrolyzing activity

and thermostability was measured after 10 min incubation in the same buffer. Figure 4 shows the optimum temperature and thermostability of the glucoamylase G1. Five glucoamylases showed the same results for optimum temperature and thermostability for activity on gelatinized starch. G1, G2 and G3 had the same optimum temperature and thermostability for activity on raw starch. The optimum temperature of G1, G2 and G3 on raw starch was 65 °C. These were higher temperatures than those for activity on gelatinized starch. We consider that the shift is due to a gelatinization effect. Five glucoamylases were stable up to 55 °C.

Molecular mass, isoelectric point and carbohydrate content

Table 2 shows the molecular masses, isoelectric points and carbohydrate contents of glucoamylases G1–G5. The molecular masses were determined by the two methods of SDS-PAGE shown in footnote of Table 2. The electrophorogram of SDS-PAGE by the method of Laemmli is shown in Fig. 2. G1, G2, G3, G4 and G5 differ from each other in the physical properties of the enzyme molecule. In particular, G4 and G5 were apparently smaller than G1, G2 and G3.

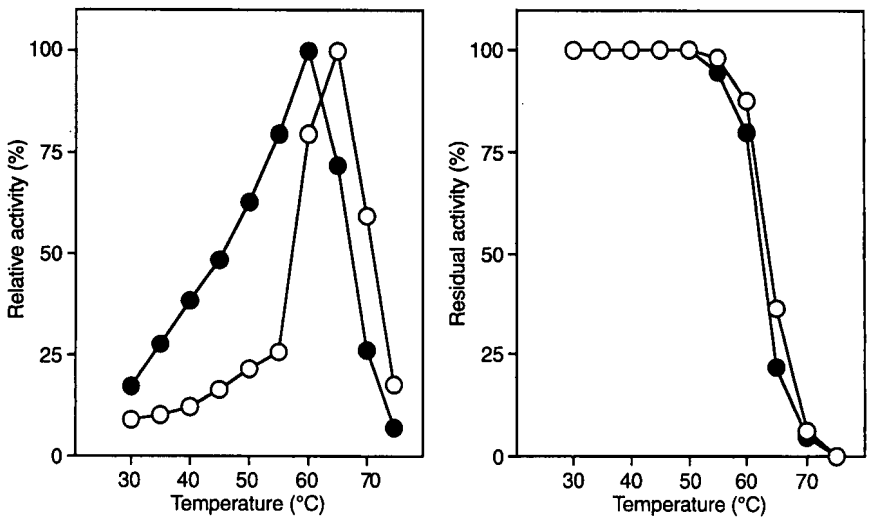

Fig. 4 Effect of temperature on the activity (*left*) and the stability (*right*) of glucoamylase G1. ○ Raw-starch-hydrolyzing activity, ● gelatinized-starch-hydrolyzing activity

Table 2 Molecular masses, isoelectric points and carbohydrate contents of glucoamylases

Parameter	G1	G2	G3	G4	G5
Molecular mass[a] (kDa)	78	78	79	70	69
Molecular mass[b] (kDa)	73	72	74	58	57
Isoelectric point	3.85	3.90	3.85	4.0	4.1
Carbohydrate content (%)	8.1	7.6	8.0	7.5	7.5

[a] Method of Weber and Osborn (1969)
[b] Method of Laemmli (1970)

Absorbance at 280 nm

The purified glucoamylases were lyophilized and weighed. The absorbance at 280 nm of 1% (w/v) protein solutions ($A^{1\%}_{1cm}$ at 280 nm) were measured. The values of $A^{1\%}_{1cm}$ at 280 nm were 17.8 (G1), 18.0 (G2), 18.1 (G3), 17.7 (G4) and 18.0 (G5).

Anomeric configuration of the product

In all five enzymes, the only product of the enzymatic hydrolysis of soluble starch was β-glucose.

Raw-starch-digesting ability

Table 3 shows raw-starch-digesting ability (Sasaki et al. 1986) of the enzymes with various types of raw starch. G1, G2 and G3 had a considerable ability to digest cereal starch, but a poor performance with root starch.

Adsorption onto raw corn starch

The adsorption of purified glucoamylases onto raw corn starch was investigated in various pH conditions. The results for G1 are shown in Fig. 5. G2 and G3 showed the same results as obtained for G1. The adsorption did not depend on pH. A 1-mg sample of glucoamylase was adsorbed 100% onto 2 g raw corn starch, and 75% onto 0.2 g.

Hydrolysis of raw starch

Figure 6 shows the time course of hydrolysis of raw starch by glucoamylase G1. Two other enzymes, G2 and G3, showed the same results as G1. Within 48 h, waxy corn, glutinous rice and nonglutinous rice were hydrolyzed at ratios comparable to those obtained when the respective gelatinized starches were used as the substrate. With tapioca, sweet potato, sago and potato, the hydrolyzing abilities of purified glucoamylases were significantly lower than those of the crude enzyme. In particular, purified glucoamylases lost the ability to hydrolyze raw potato starch.

Table 3 Raw-starch-digesting ability of the glucoamylases G1, G2 and G3 with various types of raw starch. The raw-starch-digesting ability is given as a percentage of the activity with boiled soluble starch

Starch	Raw-starch-digesting ability (%)			
	Crude	G1	G2	G3
Glutinous rice	45	32	31	30
Nonglutinous rice	62	28	29	29
Wheat	45	18	16	18
Waxy corn	38	10	12	11
Corn	28	5.7	5.0	5.1
Tapioca	29	1.6	1.9	2.0
Sweet potato	12	0.5	0.6	0.5
Sago	7.7	0.5	0.5	0.5
Potato	2.3	0.2	0.2	0.2
Boiled soluble starch	100	100	100	100

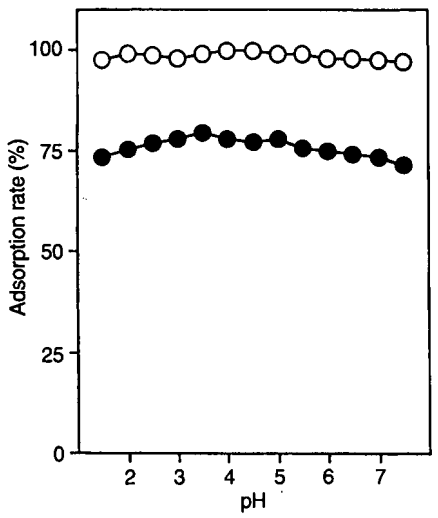

Fig. 5 Absorptions of glucoamylase G1 onto raw corn starch. Buffers used were 0.1 M HCl/KCl (pH 1.5–2.0) and 0.1 M McIlvaine (pH 2.5–7.5). ● 0.2 g starch/mg protein, ○ 2.0 g starch/mg protein

Kinetic properties

The K_m and V values of glucoamylases are summarized in Table 4. The kinetic parameters of raw-starch-digesting glucoamylases G1, G2 and G3 were quite similar to each other. Raw-starch-nondigesting glucoamylases, G4 and G5, also showed highly similar kinetic parameters. Comparing raw-starch-digesting glucoamylases with -nondigesting glucoamylases, the K_m values of the former enzymes were lower than those of the latter

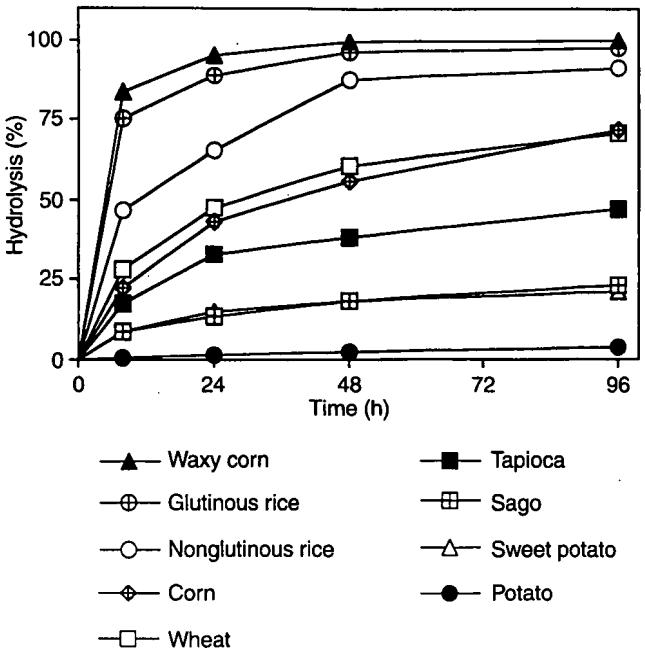

Fig. 6 Hydrolysis of various kinds of raw starch with glucoamylase G1. The reaction mixture, consisting of 0.5 ml 2% raw starch suspension in 0.1 M acetate buffer (pH 4.0) and 0.5 ml enzyme solution (10 U/ml), was incubated on a shaker at 40 °C

Table 4 Kinetic parameters of glucoamylases. The K_m values of amylose, soluble starch, amylopectin and glycogen are shown as the concentration of non-reducing terminal residues. The V values are shown as mg glucose from the non-reducing end mg protein^{-1} min^{-1}. DP the average degree of polymerization

Substrate	G1		G2		G3		G4		G5	
	K_m (mM)	V (mg mg^{-1} min^{-1})	K_m (mM)	V (mg mg^{-1} min^{-1})	K_m (mM)	V (mg mg^{-1} min^{-1})	K_m (mM)	V (mg mg^{-1} min^{-1})	K_m (mM)	V (mg mg^{-1} min^{-1})
Maltose	0.68	(0.67)	0.68	(0.67)	0.69	(0.69)	0.67	(0.83)	0.67	(0.85)
Maltotriose	0.21	(3.1)	0.21	(3.2)	0.21	(3.1)	0.23	(4.4)	0.24	(4.6)
Maltotetraose	0.095	(6.7)	0.095	(6.7)	0.088	(6.5)	0.082	(8.7)	0.083	(8.9)
Maltopentaose	0.070	(6.7)	0.071	(6.3)	0.066	(6.6)	0.067	(8.5)	0.067	(8.7)
Maltohexaose	0.048	(6.3)	0.045	(6.2)	0.050	(6.7)	0.059	(8.3)	0.060	(8.6)
Maltoheptaose	0.026	(6.3)	0.025	(6.3)	0.027	(6.1)	0.059	(8.3)	0.058	(8.4)
Amylose (DP = 18)	0.0021	(6.9)	0.0021	(6.9)	0.0022	(6.4)	0.063	(9.2)	0.065	(9.5)
Isomaltose	20.0	(0.03)	20.1	(0.03)	20.4	(0.03)	21.7	(0.043)	23.0	(0.045)
Panose	15.4	(1.2)	15.8	(1.3)	17.2	(1.3)	16.7	(1.6)	16.9	(1.6)
Soluble starch	0.0002	(6.2)	0.0003	(6.1)	0.0002	(6.7)	0.045	(8.5)	0.048	(8.7)
Amylopectin	0.0003	(6.2)	0.0002	(6.3)	0.0002	(6.4)	0.17	(7.3)	0.16	(7.5)
Glycogen	0.0007	(6.6)	0.0005	(6.3)	0.0006	(6.3)	0.63	(6.8)	0.62	(7.0)

Table 5 Amino acid compositions of glucoamylases. The composition of G2 was deduced from the cDNA

Amino acid	Composition (mol %)					
	G1	G2	G3	G4	G5	G2
Asx	10.5	10.4	10.7	11.0	9.9	10.1
Thr	11.8	11.0	11.1	11.4	10.4	10.5
Ser	12.8	11.8	12.0	11.7	11.7	11.4
Glx	7.1	7.6	7.6	7.5	7.5	7.5
Pro	2.9	3.4	3.2	3.5	3.3	3.3
Gly	7.8	8.1	8.0	7.9	8.5	8.5
Ala	11.4	11.8	11.7	11.6	12.4	12.4
Val	5.5	5.3	5.3	5.2	5.5	5.2
Cys	1.2	0.8	0.8	0.7	0.8	0.8
Met	0.5	0.8	0.8	0.8	0.8	0.8
Ile	5.5	5.4	5.4	5.3	5.0	5.1
Leu	6.1	6.3	6.3	6.2	6.7	6.7
Tyr	5.9	6.0	5.9	5.9	6.0	6.0
Phe	3.4	3.6	3.5	3.5	3.7	3.8
Trp	2.7	2.6	2.6	2.6	2.4	2.5
Lys	2.5	2.6	2.6	2.6	2.8	2.8
His	0.4	0.3	0.3	0.5	0.3	0.3
Arg	2.0	2.2	2.2	2.1	2.3	2.3

enzymes for highly polymeric substrates (e.g., amylose, soluble starch, amylopectin and glycogen). On the other hand, the *V* values of the former enzymes were slightly lower than those of the latter enzymes. This phenomenon of different *V* values was caused by differences of molecular mass, the *V* values were, in fact, the same for all five glucoamylases.

N- and C-terminal sequences of glucoamylases

The N-terminal sequences of all five glucoamylases were identical and were pGlu-Ser-Ala-Xaa-Ala-Thr-Ala-Tyr-Leu-Thr-Lys-Glu-Xaa-Ala-Val-Ala-Lys-Asn-Gly-Val. The N-terminal amino acids of these enzymes were blocked and then liberated by pyroglutamylpeptidase. The C-terminal sequences of G1 and G2 were identical and were Glu-Ser. The C-terminal amino acids of G3, G4 and G5 were not released by carboxypeptidase Y.

Amino acid compositions

Table 5 shows the amino acid compositions of five glucoamylases and compares them with that of the deduced amino acid sequence from cDNA of G2 (Nagasaka et al. 1995). There was no significant difference among the amino acid compositions of all the enzymes.

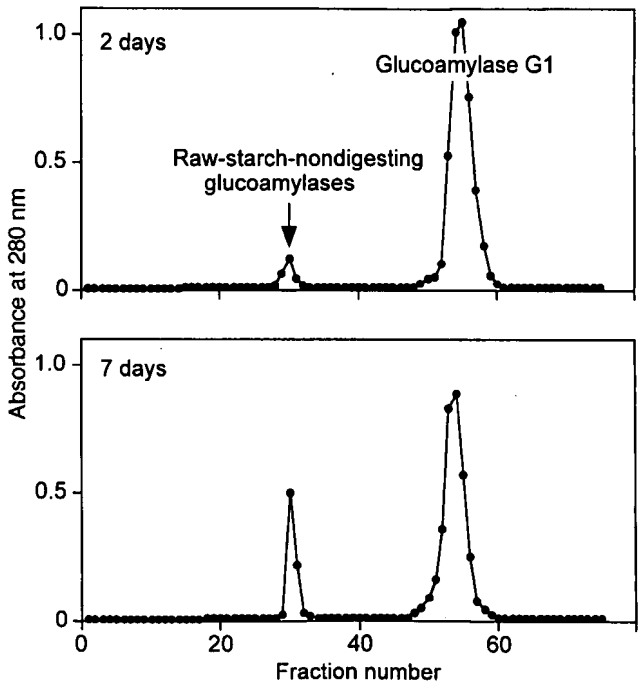

Fig. 7 DEAE-Toyopearl 650 M column chromatography of the sample of glucoamylase G1 digested with protease. Column size: 2.1 × 13 cm. Fraction volume: 8 ml. Equilibration: 10 mM acetate buffer (pH 5.0). Elution: linear gradient of NaCl (0–0.25 M). Flow rate: 30 ml/h

Protease digestion of glucoamylases

Each 600-U sample of purified glucoamylases G1, G2 and G3 was digested with 600 U *Corticium* protease preparation at pH 3.0, 30 °C for 2 days and 7 days (Fig. 7). Protease-digested glucoamylases were purified to an electrophoretically homogeneous state (data not shown) by the same method as used for the purification of G4 and G5. In the chromatofocusing step, the early-eluted fraction and the late-eluted fraction were termed G″ and G′ respectively. Table 6 shows the

Table 6 General properties of protease-digested glucoamylases

Property	Glucoamylase										
	G1	G2	G3	G1′	G2′	G3′	G4	G1″	G2″	G3″	G5
Action on raw starch	+	+	+	–	–	–	–	–	–	–	–
Adsorption onto raw starch	+	+	+	–	–	–	–	–	–	–	–
Molecular mass (kDa)	78.0	78.0	79.0	70.0	70.0	70.0	70.0	69.0	69.0	69.0	69.0
Isoelectric point	3.85	3.9	3.85	4.0	4.0	4.0	4.0	4.1	4.1	4.1	4.1
Optimum pH	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Optimum temperature (°C)	60	60	60	60	60	60	60	60	60	60	60

general properties of protease-digested glucoamylases. The characteristics of G1′, G2′ and G3′ were found to be quite similar to each other, and were the same as those of G4. G1″, G2″, G3″ and G5 also had highly similar characteristics.

Discussion

The enzyme secreted by *C. rolfsii* has high raw-starch-digesting activity. This system consisted of three forms of raw-starch-digesting glucoamylase, two forms of raw-starch-nondigesting glucoamylase and a small amount of α-amylase (Fig. 1). The amylase system of *C. rolfsii* is similar to that of other filamentous fungi. G1 and G2 are the main components of the crude enzyme and seem to play a major role in raw-starch digestion (Table 1).

The purified G1, G2 and G3 had very similar characteristics to each other, and acted strongly on cereal raw starch. On the other hand, the activities on sweet potato, sago and potato were significantly low (Table 3, Fig. 6). Upon addition of the *Corticium* α-amylase fraction, which has no activity on raw starch, the activities of purified G1, G2 and G3 on raw sago and potato starch returned to a level comparable to that of the crude enzyme (data not shown). Ueda and Kano (1975) and Abe et al. (1988) reported a similar phenomenon of α-amylase enhancement. However, the mechanism was not clarified. Also in the *C. rolfsii* amylase system, a small amount of α-amylase was required for the degradation of a few kinds of raw starch that are not easily hydrolyzed with glucoamylase.

G4 and G5 had very similar characteristics to raw-starch-digesting glucoamylases G1, G2 and G3, except for the molecular masses and action on raw starch. The amino acid sequence deduced from the cDNA of G2 showed that the motif of the starch-binding domain is located at the C terminus of the polypeptide (Nagasaka et al. 1995). Five forms of glucoamylase had the same N-terminal amino acid sequence. These results suggested that the raw-starch-digesting glucoamylases were digested by protease and were transformed to raw-starch-nondigesting glucoamylases by losing the starch-binding domain at the C terminus. By in vitro digestion with a *Corticium* protease fraction, G1, G2 and G3 all changed into two protease-digesting glucoamylases just like G4 and G5 (Fig. 7, Table 6). With the additional evidence of the amino acid composition (Table 5), it is presumed that G1, G2 and G3 were derived from identical polypeptide chains and had different carbohydrate moieties located in a region lost after protease digestion.

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